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Detection and Elimination of *Salmonella* Mbandaka from Naturally Contaminated Alfalfa Seed by Treatment with Heat or Calcium Hypochlorite[†]

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ABSTRACT

In 1999, consumption of alfalfa sprouts contaminated with *Salmonella* Mbandaka led to a multistate outbreak of salmonellosis. In this study, the implicated alfalfa seed lot (no. 8119) was confirmed to be contaminated with *Salmonella* Mbandaka at a detection frequency of approximately 72% per replicated 100 g of seed. The sensitivity of detection was improved by a combination of nonselective and selective enrichment of 5.0 ml of germination effluent, followed by immunomagnetic separation. Detection of low levels of viable cells with nonselective enrichment, employed to enhance the recovery of stressed or injured cells, was facilitated by the application of *Salmonella*-specific polymerase chain reaction (PCR). With PCR assays, *Salmonella* Mbandaka was detectable on seed stored at 5°C for at least 11 months, but at an increasingly diminishing frequency. Using conventional techniques, viable populations were detected in the seed germination effluent from seeds stored for up to 8 months. Seed treatments with buffered (to pH 7) and unbuffered solutions of calcium hypochlorite, providing approximately 2,000 and 20,000 ppm of free chlorine, for 10 min were equally effective in eliminating viable populations of *Salmonella* Mbandaka. However, aqueous heat treatments at up to 85°C for 1 min did not eliminate the naturally occurring contaminant from the seed. Reductions of >15% in germination were observed following heat treatments of 65°C for ≥6 min or 70°C for ≥4 min. On the basis of these results, aqueous heat treatments alone do not appear to be a viable alternative to hyperchlorination as an effective method to eliminate *Salmonella* from alfalfa seed.

The association of recognized or potential human microbial pathogens on fresh produce has recently been thoroughly reviewed (1, 3, 5, 21) and is a well-recognized potential source of risk. The occurrence of bacterial pathogens on raw fruits and vegetables is adequately documented on a global scale, but their presence is erratic and the frequency of isolation varies widely according to the investigator, the location, the crop, and the season (5, 22). Beuchat (1) has compiled an extensive list of documented pathogen associations and prevalences for vegetables.

FoodNet data for the five original surveillance sites indicates that the incidence of salmonellosis increased from 12.3 to 14.8 illnesses per 100,000 people (7) from 1998 to 1999. The Centers for Disease Control and Prevention noted that several 1999 outbreaks linked to unpasteurized orange juice, mamey pulp and juice, and seed sprouts may be associated with the recorded increase in the frequency of salmonellosis. Infection by *Salmonella*, represented by over 2,000 variants, rarely results in death but can cause severe stomach pain, diarrhea, nausea, and vomiting and may cause lifelong disorders.

Unlike the production processes for most fruit and veg-

etable commodities, the process of seed sprouting takes place under environmental conditions that strongly support the rapid multiplication and dissemination of *Salmonella* or other related bacterial human pathogens. Several outbreaks of foodborne illness due to the consumption of sprouts contaminated with *Salmonella* or *E. coli* O157:H7 have occurred, and outbreaks can be international in scope because of the seed distribution system (14, 15, 21, 23). For the majority of outbreaks, epidemiological evidence points to the seed as the original source of contamination, although the pathogen often cannot be isolated from the implicated seed (16). Seedborne bacteria can increase from a low frequency of contamination to a high population density within 48 h of propagation (12). Aqueous chemical seed treatments, as a primary preventive strategy used during the sprouting process, have been extensively evaluated with regard to their ability to reduce bacterial human pathogens on laboratory-inoculated seed (2, 4, 6, 9, 12, 13, 20). In response to the findings of Taormina and Beuchat (20) and the critical need for action to protect public safety, California public health officials and the Food and Drug Administration strongly recommended a uniform, voluntary industry practice of treatment of all alfalfa seed with a solution of 20,000 ppm free chlorine from Ca(OCl)₂. This treatment is currently considered the "gold standard" and is also recommended by the National Advisory Committee on Microbiological Criteria for Foods (15). A 10-min treat-

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† Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

ment results in a 2.8- to 4.5-log reduction of *Salmonella* on laboratory-inoculated alfalfa seed and does not reduce seed germination to below a commercially acceptable level (~90%) (4, 9, 20). All tests reported to date concerning the efficacy of chemical seed treatments involving calcium hypochlorite have been performed on laboratory-inoculated seed. One objective of this research was to validate the efficacy of seed treatment with calcium hypochlorite for naturally contaminated seed with conventional enrichment and polymerase chain reaction (PCR)-based techniques.

In California, the recommended application of hypochlorinated water as a seed treatment was in conflict with the certification standards of producers of organic sprouts. In part, this conflict was due to a concern about the potential for the production and environmental release of harmful chlorinated by-products such as trihalomethanes (17). In response, several organic sprout processors have explored alternative methods of pathogen elimination, relying primarily on aqueous heat treatments of various time-temperature combinations. A second objective of this study was to determine the effects of the range of the industry's commonly used time-temperature regimes on alfalfa seed germination rates and the efficacy of these treatments for the elimination of *Salmonella* from naturally contaminated seed.

MATERIALS AND METHODS

Location of studies. Unless noted, all studies were carried out at the University of California-Davis.

Sources of naturally contaminated seed. The naturally contaminated alfalfa seed (lot 8119) used in these studies either was obtained from the California Department of Health Services, Food and Drug Branch, or was purchased directly from a U.S. seed distributor. In 1999, alfalfa sprouts grown from this seed lot were implicated in a multistate (Oregon, Washington, and Idaho) outbreak of salmonellosis due to *Salmonella* Mbandaka. Upon receipt, the seed was distributed into 2-liter screw-cap plastic jars or held in the original kraft paper sacks and stored at 5°C for the duration of the study.

Detection and isolation of *Salmonella* from alfalfa seed. Initially, isolations were carried out with a modification of the procedure reported by Beuchat (2). Briefly, 25 g of seed was blended in a commercial Waring blender at the high-speed setting for 1 min in 225 ml of tryptic soy broth (Becton Dickinson, Sparks, Mass.), transferred to a sterile 1-liter flask, and placed on a reciprocal shaker at 150 rpm for 1 h at 30°C. Serial dilutions were prepared in sterile peptone water (PW; 0.1%, wt/vol) and spread plated onto bismuth sulfite (BSF) agar (Difco Laboratories, Detroit, Mich.) and xylose lysine desoxycholate agar (Becton Dickinson and Company, Cockeysville, Md.). Characteristic colonies were purified three times on BSF agar and preliminarily identified by carbon utilization patterns with the Biolog GN system (Microlog Release 3.2, Biolog, Inc., Hayward, Calif.) according to the manufacturer's instructions and with biochemical tests using BBL Enterotube II multimedia tubes (Becton Dickinson). Presumptive *Salmonella* isolates were sent to the California Department of Health Services, Microbial Diseases Laboratory, Berkeley, Calif., for serotyping and ribotyping at the Food and Drug Partnership Laboratory (Los Angeles, Calif.).

Methods for enhanced detection were evaluated when the ini-

tial experiments described above demonstrated that the level of contamination by *Salmonella* was low. These methods included both immunomagnetic selective (IMS) enrichment with Dynabeads anti-*Salmonella* (Dynal, Inc., Lake Success, N.Y.) and a PCR technique (8, 19) employed alone or in combination. Genus-specific PCR primers for *Salmonella* were designed on the basis of a primer sequence reported by Soumet et al. (19). The specificity of the final primer pair was confirmed by using known homologous and heterologous *Enterobacteriaceae* and nonenteric bacteria commonly associated with various produce items, irrigation water, and postharvest cooling water (data not reported). Additional tests, comparing presumptive positive colonies from selective enrichment cultures of various fruit and vegetable rinsates, were conducted (data not reported).

The basic method was as follows. Replicated 25-g alfalfa seed samples were imbibed in sterile 0.5-liter flasks containing 200 ml of sterile PW. Each separate test involved 10 individual flasks and one additional flask of seed inoculated with *Salmonella* Typhimurium strain LT2 rif^r (resistant to at least 80 mg of rifampicin per liter and 100 mg of nalidixic acid per liter). *Salmonella* Typhimurium LT2 was used to develop the technique because the selectivity of rifampicin-amended media greatly facilitated the recovery of small populations in the presence of high population densities of competitive bacteria from alfalfa seed that co-enrich with *Salmonella* Mbandaka. The inclusion of an inoculated control strain permitted the simultaneous comparison of viable-cell enrichment and PCR detection, which has the potential to detect both viable and nonviable populations following disinfection treatments. Standard suspensions of washed cells were prepared from stationary phase cultures grown in Luria broth (LB; Difco) and were added to establish positive control seed flasks with final starting concentrations of 1.6 log₁₀ CFU/ml. Seeds were allowed to germinate at 37°C for 18 h on a reciprocal shaker (200 rpm). From each replicate flask, 100 µl of seed germination fluid was transferred into 4.0 ml of LB and subsequently shaken (250 rpm) at 37°C. After 3 h, 200 µl of broth was transferred to 9.0 ml of Rappaport-Vassiliadis R10 (RV; Difco) broth and incubated without shaking for 18 h at 37°C. A loopful of this selective enrichment broth was streak plated onto BSF and xylose lysine desoxycholate agar as previously described. Characteristic colonies were purified at least twice on BSF agar and preliminarily identified with the Biolog GN system as described above. In addition, 2 ml of the primary LB was concentrated by centrifugation, and the pellet was suspended in 50 µl of sterile distilled water. Alternatively, 20 µl of Dynabeads was added to 1.0 ml of the primary LB and processed with a magnetic particle concentrator according to the manufacturer's instructions. The resultant sample tube was placed in a boiling water bath for 5 min to lyse cells. A 1-µl aliquot of the final solution was used as the PCR reaction template.

Modifications to the basic protocol for combined IMS-PCR detection were used following various heat and chlorine seed treatments to increase the probability of detecting low-level survivors in germination media. Following heat or chlorine seed treatments, as described below, 2.0 to 5.0 ml of germination fluid was removed, added to a proportional volume (1:40) of LB, and incubated as described above. All other procedures were as described.

Calcium hypochlorite treatments. For experiments carried out at the Eastern Regional Research Center (ERRC), 100 g of seed was placed into 500-ml sterile beakers and rinsed twice with 200 ml of sterile tap water by stirring constantly with a sterile spatula by hand for 2 min per rinse. After the removal of the final

rinsate, 3% (wt/vol) calcium hypochlorite (Aldrich) was added, and the seeds were agitated by hand for 10 min. Calcium hypochlorite (12 g) was added to 400 ml of buffer (500 mM potassium phosphate, pH 6.8) or sterile tap water at 22°C and stirred for 20 min. These solutions provided approximately 20,000 ppm of free chlorine, as determined with Reflectoquant test strips for free chlorine and reader (EM Science, Gibbstown, N.J.). As appropriate, sterile buffer or tap water alone was used as a control. Four or five experiments were carried out with buffered and unbuffered solutions, respectively, with duplicate seed samples for each treatment and for controls. The treated seeds were rinsed twice with 200 ml of sterile tap water each time as described above; 500 ml of lactose broth was added, and the seed mixture was transferred to a sterile stainless steel blender receptacle. The mixture was homogenized for 1 min, and the homogenate was transferred to a filter-lined stomacher bag and incubated for 18 h at 37°C without shaking. Samples (1 ml) were removed and placed into tubes containing 10 ml of either tetrathionate broth (Difco) or RV (Difco) broth; four tubes of each broth were inoculated with samples from each stomacher bag. The inoculated tubes of selective broth media were incubated without shaking for 18 h at 37°C (tetrathionate broth) or at 42°C (RV broth). After incubation, 50- μ l samples from each tube were streaked onto single plates of Hektoen enteric (HE; Difco) agar or XLT-4 agar (Difco), and the inoculated media were placed at 37°C. The plates were examined after 24 and 48 h for the presence of black colonies typical of *Salmonella*.

In addition to broth enrichment, experiments concerning the detection of survivors on sprouts obtained from treated seed were also carried out. For these experiments, seeds were rinsed and treated with calcium hypochlorite (prepared in tap water only) as described above. After the final two rinses with sterile tap water, the seeds were transferred to sterile glass trays (27 by 38 by 5 cm) lined with germination paper. Sterile tap water (50 ml) was added to each tray, and the trays were covered with aluminum foil and placed on the lab bench at room temperature (22°C). Equal amounts of sterile tap water (50 to 100 ml) were added daily to each tray. After 5 days, 100-g samples of the grown sprouts (collected at random from several regions of each tray) were placed into sterile stainless steel receptacles. Lactose broth (500 ml) was added and the sprouts were homogenized for 1 min. The homogenate was transferred to sterile stomacher bags, and after 1 h of incubation at room temperature (22°C) the pH was adjusted to approximately 7 with 5 N NaOH. Aliquots (1 ml) of the homogenates were placed into 10 ml of the selective broth media (tetrathionate and RV broth; four tubes of each medium per sample) and incubated for 20 h at either 37 or 42°C, respectively, without shaking (direct selective enrichment). The remainder of the sample in the stomacher bag was incubated overnight at 37°C, and then 1.0-ml samples were transferred to the two selective broth media (nonselective preenrichment followed by selective enrichment) as described above. After incubation, 50- μ l samples from each of the tubes of selective broth media were streaked onto single plates of both HE agar and XLT-4 agar, and the plates were incubated at 37°C for up to 48 h.

For all experiments, black colonies typical of *Salmonella* were streaked onto HE agar or XLT-4 agar three times to obtain pure cultures. The pure cultures were then subjected both to slide agglutination tests with Bacto *Salmonella* O antiserum poly A-I and Vi (Difco) and to biochemical tests with BBL Enterotube II multimedia tubes (Becton Dickinson). For serotype determination, presumptive *Salmonella* isolates were submitted to the Animal and Plant Health Inspection Service National Veterinary Services Laboratory, Ames, Iowa, for evaluation.

In parallel studies at the University of California-Davis,

solutions of calcium hypochlorite (InduClor, PPG Industries 65% active ingredient) providing approximately 2,000 and 20,000 ppm of free chlorine were evaluated with regard to their efficacy. The formulated active ingredient was verified by carrying out dilutions in sterile distilled water to within the range of accuracy for total and free chlorine using a chlorine titrometer (CL Titrimeter 397; Fisher Scientific). To simulate commercial practice, solutions were prepared in tepid tap water (~35°C) by slowly stirring 3.3 and 33 g/liter for 10 min to dissolve granules. Replicated ($n = 10$ to 16) 25-g samples of seed were placed in 300 ml of sterile tap water or in the hypochlorite solutions and held for 30 and 15 min for the 0.2 and 2.0% solutions, respectively, with occasional stirring. Following treatment, the seeds were washed twice with threefold volumes of sterile tap water. After the alfalfa seeds were washed, they were imbibed in sterile 0.5-liter flasks containing 200 ml of sterile PW, as described above. Seeds were allowed to germinate at 37°C for 18 h on a reciprocal shaker at 200 rpm prior to sampling for qualitative detection, as described above.

Hot water treatments. For the initial set of experiments, seeds were removed from refrigerated storage and allowed to equilibrate to room temperature (24°C) prior to treatment. Two hundred seeds were placed in prewetted no. 3 filter paper (Fisher Scientific, Pittsburgh, Pa.) envelopes and immersed in a water bath held at 60, 65, or 70°C for up to 10 min. As a control, for each time interval a single filter paper envelope containing 200 seeds was immersed in ambient-temperature water (24°C). Treated seeds were allowed to cool to ambient temperature, and the percentage of germination was determined as described below.

Reflecting the protocols for heat treatments reportedly used in California by some commercial organic sprout producers, additional experiments with 25 g per replicated test were conducted. These experiments included a preheating seed soak step followed by hotter, shorter heat treatments. Seeds were removed from cold storage as 25-g aliquots in a sterile disposable capped plastic beaker and allowed to reach ambient temperature. Sufficient 55°C sterile water was rapidly added to cover the seeds, and the beakers were placed in a 55°C water bath for 40 min. Beakers were held in place on a weighted rack support such that the beaker water line was several millimeters below the water bath level. The pre-soak liquid was decanted, and an equal volume of 80 or 85°C sterile water was added and allowed to contact the seed for 1 min. After removal of the treatment liquid, an equal volume of 25°C sterile water was added and left for 10 min prior to decanting to promote cooling.

For comparison, seeds of lot 8119 were inoculated with a high level of *Salmonella* Typhimurium by the immersion of 25 g in a suspension of 6.0 log₁₀ CFU of *Salmonella* Typhimurium LT2 rifal per ml for 4 h. Inoculated seed was spread on a sterile plastic tray, covered with double-layer cheesecloth to minimize aerosol transfer, and dried at ambient temperature (~24°C) for approximately 40 min. Seeds were visibly dry at this time. The starting density of the inoculum on the seed was approximately 10³ CFU/g, as indicated by standard dilution plating onto BSF agar, xylose lysine desoxycholate agar, LB, or standard methods agar amended with 80 mg of rifampicin per liter. Seeds were treated with hot water as described above.

After heat treatments, the presence of surviving *Salmonella* was determined by the standard protocol of nonselective broth preenrichment followed by selective broth enrichment followed by dilution plating onto selective agar media as described above. Selected black colonies typical of *Salmonella* growth on BSF agar were confirmed to be *Salmonella* as described above.

Additional tests were conducted in an attempt to increase the sensitivity of detection of potential survivors and resuscitated heat-stressed cells. Heat treatments at 80 and 85°C were repeated as described above. Treated seeds were placed in 200 ml of sterile PW and allowed to germinate at 37°C for 18 h with shaking at 200 rpm, and 2.0 ml of germination fluid was transferred to 2.0 ml of LB. Simultaneously, 5.0 ml of the same solution was transferred to 50 ml of RV media and selenite cysteine broth (Difco). Samples were incubated for 9 h at 37°C with shaking at 150 rpm. Six more 1.5-ml aliquots were centrifuged, and the resultant pellets were washed, pooled, centrifuged, and resuspended in 50 µl of sterile distilled water and prepared for use in PCR analysis. A 1-µl sample was used as the PCR template.

Determination of seed germination percentage. To determine the germination percentage, treated seeds (100 per replicate) were placed on agar (1.5% granulated agar, Difco) in 150-mm plastic culture dishes, or 200 seeds were placed on top of two sheets of prewetted no. 3 filter paper. Seeds were spread out on the agar plates or in envelopes made from the filter paper. The filter paper envelopes were placed in sterile glass cylinders with a small amount of deionized water at the base to maintain uniform moisture and were covered with aluminum foil. The seeds were incubated at approximately 24°C for 48 h. Evaluations were based on 200 to 400 seeds for each exposure time-temperature combination. A seed was considered to have germinated if the seed coat was broken and a visible sprout was extending at least 8 mm from the original seed. Swollen seeds or ruptured seeds with sprout tissue still inside were not counted as true sprouts, because it was noted that such seeds rarely sprouted completely after an additional 24 h of incubation.

Statistical analysis. For data generated at the ERRC, statistical analyses were carried out on arcsine square root transformations of the binomial response (18). For data generated at the University of California-Davis, a two-sample Z test was used to test for the difference between two populations (filter paper versus agar) for each of the two separate germination experiments. The level of significance was 0.05. Two-sample Z tests were used to test for the difference between population proportions (germination percentages) for the control groups (time = 0) versus increasing times of exposure (at each temperature) until a statistically significant ($\alpha = 0.05$) difference was found.

RESULTS AND DISCUSSION

Detection of natural contamination of alfalfa seed lot 8119. Because of differences in the numbers of 25-g replicates used in each experiment, isolation rates were sometimes normalized to 100 g of sample. With conventional techniques of nonselective preenrichment and selective broth enrichment followed by plating onto selective agar media, viable *Salmonella* contamination in seed lot 8119 was detected in approximately 67% of the pooled 100-g samples. With the combined IMS-PCR technique, approximately 72% of the pooled 100-g seed samples tested positive, as indicated by the amplification of the PCR product size that matched that of the amplification product of the seed-inoculated positive control *Salmonella*. The identification of selected black colonies on selective agar media as *Salmonella* Mbandaka was confirmed by the results of the carbon utilization assays and the biochemical tests, as well as serology and ribotyping.

Natural contamination of alfalfa seed with *Salmonella*

is presumed to be limited and randomly distributed. Levels of *Salmonella* from three different outbreak-associated lots were determined to be 0.034 to 1.8 most probable number/100 g (10), indicating the need for a sample size larger than the traditional 25 g when this product is tested.

The application of immunomagnetic separation alone or in combination with *Salmonella*-specific PCR primers confirmed the detection of low levels of viable *Salmonella* cells in alfalfa seed lot 8119 (Fig. 1). Fach et al. (8) established a minimum threshold of 6.0 log CFU/25 g for the detection of dead *Salmonella* cells in various food samples. Initial viable cell densities in lot 8119 were presumed to be far below this level on the basis of the low recovery by standard enrichment procedures. Earlier studies (24) were conducted to demonstrate that heat-killed cells of *Salmonella* Typhimurium LT2 rif^r were not detected in this system if the starting population density was less than 1.47 to 1.69 log CFU/ml or 3.0 log CFU/25 g of seed. Following placement in 200 ml of PW for germination, removal of 100 µl after 18 h, and dilution in 4.0 ml of LB, the concentration of initial inoculated cells would have been reduced to less than -0.9 log CFU/25 g. Although unlikely, the possibility of PCR detection of nonviable cells or *Salmonella* DNA cannot be unequivocally ruled out by the procedures used in this study. Of 18 initial 25-g samples tested, only six were presumably positive on the basis of the appropriate PCR band size and the typical colony appearance on BSF agar. The detection technique that led to the greatest detection efficiency by PCR involved relatively large volumes of germination fluid (5 ml in 195 ml of LB) in combination with IMS enrichment and increased the frequency of detection to approximately 72% (23 of 32 of the 25-g seed samples tested positive).

With conventional isolation methods, Inami and Moler (11) and Inami et al. (10) compared sprouting and shredding techniques for the detection of *Salmonella* from naturally contaminated alfalfa seeds. The sprouting method involved incubation of water-soaked seeds for 4 days at 20 to 24°C, while the shredding method required grinding of the dry seed into a coarse powder in a blender prior to conventional isolation methods. In the current study, a modified germination technique was used prior to the application of conventional and IMS-PCR techniques. While not directly compared with other isolation methods, the technique described here eliminates the use of blenders (whose sterilization can be time-consuming), shortens the germination period from 4 days to 18 h, and eliminates the need for additional manipulation of the seed.

Effect of seed storage at 5°C on recovery of *Salmonella* Mbandaka. The survival of naturally contaminating *Salmonella* in seed lot 8119 was reduced, but not eliminated, by seed storage at 5°C over an 18-month period. The observed frequency of detection dropped from approximately 70 to 25% of replicated 25-g samples ($n = 24$) during the period of evaluation, as determined by combined IMS-PCR and qualitative conventional nutrient media enrichment and plating. Confirmed viable cells were detected by enrichment culture and IMS-PCR following storage at

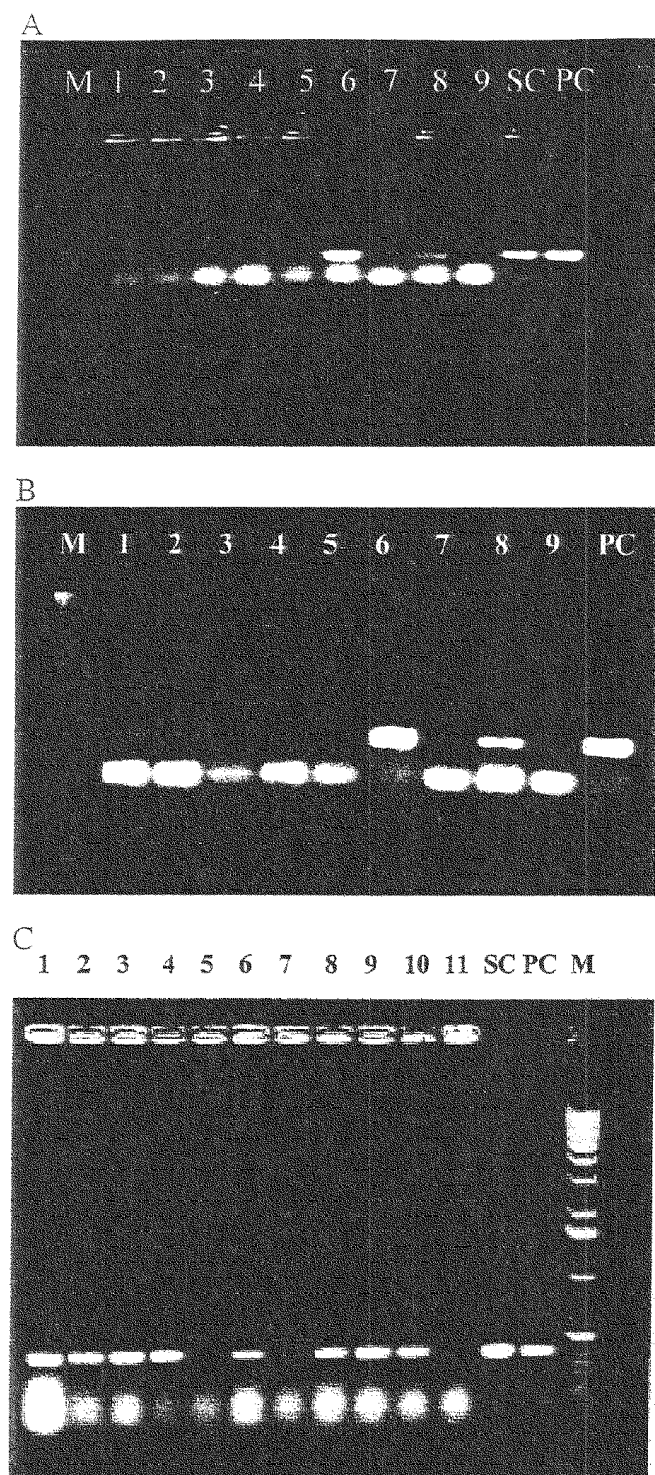


FIGURE 1. Detection of natural *Salmonella* contamination on alfalfa seed by PCR. (A) Replicated groups of 25-g alfalfa seed samples from bulk lot 8119 were used for each independent assay. Seed germination fluid was transferred to nonselective enrichment media for 18 h to minimize the likelihood of PCR detection of nonviable *Salmonella* cells. M, marker; SC, lot 8119 inoculated with *Salmonella* Typhimurium LT2 rif^r; PC, pure culture of *Salmonella* Typhimurium LT2 rif^r in distilled water. Lanes 1, 6, and 8 were considered positive. Viable cultures of *Salmonella* Mbandaka were obtained from group 6. (B) Duplicate samples from panel A processed with anti-*Salmonella* immunomagnetic enrichment. Additional weak PCR products of the expected size appear in lanes 2 and 4. (C) In a larger set of 25-g seed samples ($n = 32$), a higher frequency of detection was obtained by mixing 5 ml of germination fluid in 195 ml of LB and incubating it at

5°C after 18 months in germinating seed effluent. Others have reported detection of *Salmonella* in naturally contaminated alfalfa seed by conventional techniques after storage at room temperature for 2 years (10).

Efficacy of seed treatments with calcium hypochlorite. At the ERRC, preliminary investigations involved 25-g samples of the naturally contaminated seed treated with 100 ml of 3% (wt/vol) calcium hypochlorite prepared in buffer. Three experiments were run, with three replications per treatment per experiment. Out of a total of nine seed samples treated with the buffered calcium hypochlorite solution, no black colonies typical of *Salmonella* were observed. Only one the nine control samples produced black colonies on the selective agar media. Two black colonies were streaked for purity and were confirmed to be *Salmonella* by slide agglutination and biochemical tests. Because the untreated samples showed no positives, the numbers of positives in the untreated controls were analyzed to determine if they were statistically nonzero, thus differentiating the results for the untreated samples from those for the treated samples. The numbers of positives for the untreated samples were significantly nonzero at $P = 0.01$.

Since so few control samples consisting of 25 g of seed were found to be positive for *Salmonella*, further testing was done with the seed sample size being increased to 100 g. Both buffered and unbuffered solutions of calcium hypochlorite were used for these trials. Four experiments were conducted with buffered or unbuffered solutions, with duplicate seed samples for each treatment. A 10-min treatment with 3% (wt/vol) calcium hypochlorite prepared in buffer resulted in no black colonies being observed on the selective media for all eight seed samples tested. For the buffer controls, three of eight seed samples resulted in black colonies on the selective agar media. A 10-min treatment with 3% (wt/vol) calcium hypochlorite prepared in tap water also resulted in no black colonies on the selective agar media for all 10 seed samples, while 2 of the 10 seed samples treated with only tap water tested positive for black colonies. For each treatment that showed the presence of black colonies on the selective agar media, two black colonies were streaked to purity and confirmed to be *Salmonella*. Statistical analyses indicated that the results for both the buffer and the tap water controls were significantly nonzero at $P = 0.05$.

In addition to testing seeds for surviving *Salmonella* immediately after treatment with chlorine, we also tested sprouts grown from the treated seeds. Results for the samples of sprouts grown from seeds treated with sterile tap water alone (controls) were very consistent with one of three sprout samples confirmed to harbor *Salmonella* Mbandaka for each of the three experiments (for a total of three

37°C for 18 h. Enrichment by anti-*Salmonella* IMS was carried out prior to PCR assays. M, marker; SC, lot 8119 inoculated with *Salmonella* Typhimurium LT2 rif^r; PC, pure culture of *Salmonella* Typhimurium LT2 rif^r in distilled water. Lanes 1 through 4, 6, and 8 through 10 were considered positive.

TABLE 1. Germination rates (%) of alfalfa seed heated for various times and to various temperatures

Time (min)	Temperature (°C) ^a		
	60	65	70
0	97.5	92.0 (4.4)	93.7 (3.3)
1	ND ^b	91.3 (0.6)	92.0 (3.0)
2	98.0	89.0 (3.6)	89.2 (2.5)
4	98.5	84.7 (2.1)	73.8 (3.1)
6	96.5	75.3 (4.0)	54.5 (6.3)
8	96.5	58.0 (8.9)	34.3 (4.1)
10	96.5	45.0 (6.9)	25.3 (9.6)

^a Numbers in parentheses are standard deviations ($n = 3$ to 6 samples of 100 seeds). A single 200-seed sample was used to determine germination rates at 60°C.

^b ND, not determined.

positives out of a total of nine samples). The control samples were significantly nonzero ($P < 0.05$) on the basis of correction for continuity and a one-sided test. *Salmonella* Mbandaka was detected in the homogenized samples both by direct selective enrichment and by preenrichment followed by selective enrichment. However, the number of resultant black colonies on the selective agar media was much larger when samples had undergone both preenrichment and selective enrichment. None of the nine samples of sprouts grown from seeds treated with calcium hypochlorite resulted in black colonies on selective media with or without the preenrichment step being included in the protocol followed for detection.

Additional tests with both 2,000 and 20,000 ppm of free chlorine from calcium hypochlorite were conducted at the University of California–Davis. The results of these tests demonstrated that treatment with both levels of free chlorine were effective in eliminating *Salmonella* Mbandaka from the seeds, as indicated both by the standard protocol, consisting of nonselective enrichment followed by selective enrichment and plating on selective agar medium, and by IMS enrichment and PCR techniques. Even the most inclusive detection protocol, involving 5 ml of germination fluid added to 195 ml of LB and a combination of IMS enrichment and PCR assay, did not detect any surviving pathogens.

Effect of heat treatments. The germination percentage did not significantly decline ($P = 0.28$) when seeds were held for up to 10 min at 60°C (Table 1). When seeds were held at 65°C, germination declined over the entire treatment period but was significantly reduced ($P = 0.0026$) only after a 4-min period. When seeds were held at 70°C, significantly lower germination percentages ($P = 0.0027$) were observed after 2 min but not after 1 min. Similar declines in germination rates were observed by Jaquette et al. (11). However, they observed a 50% reduction in germination after seeds were soaked for 10 min at 60°C. Variations could be due to differences in seed lots.

Heat treatments applied by some organic sprouters (80°C for 1 min) were not effective in eliminating natural contamination. Of ten 25-g samples tested, two tested pos-

itive with IMS-PCR methods. After a heat treatment of 85°C for 1 min, one of eight replicate 25-g samples tested positive with IMS-PCR methods. This heat treatment is known to be very deleterious to most seed lots. Approximately 12% of replicates from this treatment were shown to have residual populations that were detected by IMS-PCR and, assuming that viable cells were the basis of detection, would be likely to grow to high levels during a typical sprouting process. Longer sublethal presoak periods (40 to 55°C for ≥ 2 h), as sometimes evaluated in pilot commercial trials, were observed to increase the frequency of positive detection (data not presented) relative to shorter presoak times.

CONCLUSIONS

Treatment of the naturally contaminated alfalfa seed with 2,000 and 20,000 ppm free chlorine prepared in buffer or tap water eliminated *Salmonella* Mbandaka, as demonstrated by sampling of the treated seed, sampling of sprouts grown from the treated seed, and sampling of the germination fluid. Clearly, seed lot differences and variation in natural contamination population density and spatial distribution on and within seeds will influence the outcome of any treatment procedure (4, 10). In contrast, heat treatment regimes used by some organic sprout growers did not eliminate *Salmonella* Mbandaka from the naturally contaminated seed. Heat treatments causing unacceptable loss of viability in alfalfa seed did not result in pathogen elimination according to the results of this study.

A *Salmonella* detection method involving IMS-PCR after a short nonselective germination period gave results that were comparable to those of the conventional method; however, the time required for confirmation was significantly reduced.

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